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Abstract

Oestrogens can affect expression of genes encoding steroidogenic enzymes in fish gonads. However little information is available on their effects at the protein level. In this context, we first analyzed the expression of key steroidogenic enzyme genes and proteins in zebrafish testis, paying attention also to other cell types than Leydig cells. Gene expression was analysed by quantitative PCR on fluorescence activated cell sorting fractions coupled or not to differential plating, while protein synthesis was studied by immunohistochemistry using specific antibodies against zebrafish Cyp17a1, Cyp19a1a and Cyp19a1b. Furthermore, we have evaluated the effect of oestrogen treatment (17β-œstradiol, 10 nM) on the localization of these enzymes after 7 and 14 days of *in vivo* exposure in order to study how œstrogen-mediated modulation of their expression is linked to œstrogen effects on spermatogenesis. The major outcomes of this study are that Leydig cells express Cyp17a1 and Cyp19a1a, while testicular germ cells express Cyp17a1 and both, Cyp19a1a and Cyp19a1b. As regards Cyp17a1, both protein and mRNA seem to be quantitatively dominating in Leydig cells. Moreover, 17β-œstradiol exposure specifically affects only Leydig cell Cyp17a1 synthesis, preceding the disruption of spermatogenesis. The œstrogen-induced suppression of the androgen production capacity in Leydig cells is a major event in altering spermatogenesis, while germ cell steroidogenesis may have to be fuelled by precursors from Leydig cells. Further studies are needed to elucidate the functionality of steroidogenic enzymes in germ cells and their potential role in testicular physiology.

Keywords: steroidogenic enzymes, zebrafish, testis, FACS, immunolocalization, oestradiol

1. Introduction

Steroid hormones are key regulators of growth, development and reproduction in vertebrates. Sex steroid hormones are mainly produced in the gonads and their plasma concentrations change greatly during the process of gonad maturation. In fish, testis tissue produces all three major types of sex steroid hormones, progestagens, androgens, and oestrogens, each having distinct functions in the regulation of spermatogenesis, from spermatogonial stem cell self-renewal to sperm maturation, while exerting regulatory effects either directly on the testis, or indirectly via feedback effects on the brain and/or pituitary (Schulz *et al.*, 2010). However, androgens clearly are the quantitatively dominating steroid product of the testis (Schulz et al., 2010).

The synthesis of steroid hormones from a common precursor, cholesterol, is supported by numerous enzymes including several P450 cytochromes (Baroiller *et al.*, 1999). Among these P450 cytochromes, the 17-alpha-hydroxylase, 17,20 lyase (Cyp17) catalyzesthe hydroxylation of pregnenolone to 17-alpha-hydroxypregnenolone or of progesterone to 17-alpha-hydroxyprogesterone, and the cleavage of the C17,20 bond to convert 17-alpha-hydroxypregnenolone and 17-alpha-hydroxyprogesterone to dehydroepiandrosterone and androstenedione, respectively. An *in silico* study revealed the existence of two *cyp17* genes (*cyp17a1* and *cyp17a2*) in the zebrafish genome (Zhou *et al.*, 2007). Aromatases (Aromatase A (Cyp19a1a) encoded by the *cyp19a1a* gene and aromatase B (Cyp19a1b) encoded by the *cyp19a1b* gene) catalyze the conversion of androgens into œstrogens. Similar to mammals, both, Cyp17 and Cyp19a1 proteins are strongly expressed in the Leydig cells of the testes of several species of fish (Dalla Valle *et al.*, 2002a; Halm *et al.*, 2003; Yu *et al.*, 2003; Blazquez and Piferrer, 2004; Wang andOrban, 2007) including zebrafish (*Danio rerio*) (De Waal *et al.*, 2009; Hinfray *et al.*, 2011). Initially, Cyp17 was thought to be confined to classic steroidogenic tissues but mRNA, protein and activity for this enzyme were also detected in other tissues, such as brain (Hojo *et al*., 2004),

liver (Katagiri *et al*., 1998) and stomach, duodenum and kidney for rodents (Dalla Valle *et al*., 2002b). A wide distribution of this enzyme in different tissues also in other species, like zebrafish, has been demonstrated (Wang and Ge, 2004). Recently, studies in mice detected Cyp17 in germ cells; spermatogonia, pachytene spermatocytes, spermatids and sperm (Qiang *et al*., 2003; Liu *et al*., 2005), and the loss of this enzyme is associated with sperm abnormalities and infertility (Liu *et al*., 2005). This suggests that in addition to steroidogenesis, Cyp17 may have a role in sperm structure and function (Liu *et al*., 2005). Cyp19 (P450 aromatase) has also been found in a wide variety of tissues in addition to gonads: placenta, bone, adipose tissue, blood vessels, skin, endometrium and brain (see review in Chumsri *et al*., 2011). In the different mammalian species studied so far, P450 aromatase expression has been detected in several testicular somatic cells (e.g., Leydig cells) as well as in gonocytes, spermatogonia, spermatocytes (preleptotene/pachytene), spermatids and spermatozoa (see review in Chumsri *et al*., 2011). However, there is little information available in fish with respect to the question if other testicular cell types express these enzymes as well.

Testicular steroidogenesis is very sensitive to exogenous steroids. Effects following *in vivo* exposure of African catfish to testosterone include a loss of the 17,20 lyase but not of the 17-alpha-hydroxylase activity, and a reduction to 50% of the number of mitochondria and of the cytoplasmic area in Leydig cells (Cavaco *et al.*, 1999; Schulz *et al.*, 2008). In adult zebrafish, exposure to 17-beta-œstradiol (E_2) *in vivo* led to an interruption of spermatogenesis following a down-regulation of testicular androgen synthesis, presumably via feedback inhibition of gonadotropin release (De Waal *et al.*, 2009). Several other studies have also documented the inhibitory effect of œstrogens on the testicular expression of steroidogenic enzymes such as cyp17, P450 side chain cleavage, 11-beta-hydroxylase in fish (Govoroun *et al.*, 2001; Baron *et al.*, 2005; Filby *et al.*, 2006; Brion *et al.*, 2008; De Waal *et al.*, 2009) which could explain the deleterious effects of these compounds on fish reproduction (Brion *et al.*, 2004).

In this context, the aim of this study was first to localize the cellular sites of synthesis of key steroidogenic enzymes (Cyp17, aromatase A and aromatase B) involved in the production of androgens and œstrogens in zebrafish testis, paying attention also to other cell types than Leydig cells. Enzyme protein localization was studied immunohistochemically, using specific antibodies against zebrafish Cyp17a1, Cyp19a1aand Cyp19a1b. Furthermore, we have evaluated the effect of œstrogen treatment on the localization of these enzymes in order to study how deregulation of their synthesis is linked to E_2-disrupted spermatogenesis. The major outcomes of this study are that Leydig cells express Cyp17a1and Cyp19a1a, while testicular germ cells express Cyp17a1 and both, Cyp19a1a and Cyp19a1b. Moreover, E_2 exposure specifically affects only Leydig cell Cyp17a1 synthesis, preceding the disruption of spermatogenesis.

2. Materials and methods

2.1. Zebrafish maintenance

Adult (> 90 days post fertilization) wild type zebrafish (AB strain) were bred in a laboratory facility at INERIS (Institut National de l' Environnement Industriel et des Risques, Verneuil-en-Halatte, France). They were maintained in 3.5 L aquaria in a recirculation system (Zebtec, Tecniplast, Buguggiate, Italy) on a 14:10 light:dark cycle at a temperature of 25.1 ± 1.0°C. Animal culture, handling and experimentation were approved by the INERIS life science ethical committee.

2.2. Zebrafish exposure

Adult male zebrafish were exposed to 10 nM of E_2 (Sigma-Aldrich, St Louis, MO, USA) or to solvent alone (dimethylsulfoxide (DMSO), 0.00001%; Sigma-Aldrich) during 7 and 14 days. Fish were exposed under semi-static conditions in 4 L tanks with 100% water renewal every day (temperature: 28 ± 1°C; pH: 8.2 ± 0.2; dissolved oxygen: 6.4 ± 0.2 mg/L; conductivity: 269 ± 37 µS/cm). Exposure was performed in one tank per condition, each containing 6 male fish. At the end of exposure, zebrafish were euthanized in ice-cold water, weighed and measured. Testes were removed and weighed to determine the gonadosomatic index (GSI = testis wet weight / total fish wet weight $\times$ 100).

2.3. Expression of cyp17a1 and cyp19a1 genes in sorted testicular cell fractions

To generate data on the cellular localization of testicular *cyp17a1* and *cyp19a1* expression with an independent approach, we used testes from *vasa::egfp* zebrafish (Krøvel and Olsen, 2002) as tissue donors. Testis tissue from transgenic fish was digested with 0.2% collagenase and 0.12% dispase as described previously (Nóbrega *et al*., 2010). The resulting cell suspension was immediately submitted to fluorescence activated cell sorting (FACS) using an in Flux cell sorter (BD Bioscience, San Jose, CA, USA, www.bdbiosciences.com).

In some cases, the testicular cell suspension was also submitted to a differential plating method (Luo *et al*., 2006) before FACS. Using this method, somatic cells adhere to the bottom of the plate while germ cells either remain in suspension after 2-3 days of culture, or are only loosely associated with the firmly adhering somatic cells (Fig.1). Then, FACS settings were adjusted to sort Egfp positive and negative cells from total *vasa::egfp* testicular cell suspension and also from floating/loosely associated *vasa::egfp* cells after differential plating (Fig.2). Autofluorescence was eliminated through the FACS dot plot profile generated with a testicular cell suspension from wild-type males (Fig.2A). Then, Egfp positive and negative cells were collected into tubes, centrifuged in PBS (phosphate buffer saline pH 7,4) for 10 min at 900 rpm, and immediately submitted to RNA extraction using RNAqueous ® - Micro Kit (Ambion, Austin, TX, USA, http://www.ambion.com) as described before (Nóbrega *et al*., 2010). After cDNA synthesis, the threshold cycle (Cq) values of two endogenous reference genes (*elongation factor 1-alpha, ef1α*; *18S rRNA*) and of the target genes were determined by qPCR. Primers used for the quantification of *18S rRNA* were provided by the Eukaryotic 18S rRNA TaqMan® Assay (20X mix of two unlabeled PCR primers and a FAM™ dye-labeled 18S TaqMan® MGB probe; Applied Biosystems). The target genes were: cytochrome P450, family 17, subfamily A, polypeptide 1 (*cyp17a1*) (García-López *et al*., 2010), cytochrome P450, family 19, subfamily A, polypeptide 1 (*cyp19a1a*) (Chiang *et al.,* 2001), steroidogenic acute regulatory protein (*star*) (García-López *et al*., 2010), insulin-like 3 *(insl3)* (Leydig cell-specific gene) (Good-Avila *et al*., 2009), gonadal soma-derived growth factor (*gsdf*) (Sertoli cell specific gene) (Gautier *et al*., 2011) and piwi-like2 (*piwil2*) (germ cell specific gene) (Houwing *et al*., 2008). All qPCRs were performed in 20 µl reactions, containing 10 µl Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.9 µM forward and 0.9 µM reverse primers (Supplemental Table 1) and 5 µl cDNA for each gene. For the endogenous reference genes (*ef1α*; *18S rRNA*), qPCR reactions were done in 20 µl reactions, containing 10 µl TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.3 µM forward and 0.3 µM reverse primers

(Supplemental Table 1) and 5 µl cDNA. For *18S rRNA* cDNA was first diluted to 1:200 in RNAse free water. Relative mRNA levels were determined using the 7900HT Fast Real-Time PCR System (Applied Biosystems), using default settings. Ct values of the target genes (*cyp17a1*, *cyp19a1*, *star*, *insl3*, *gsdf* and *piwil2*) were normalized with the Ct values of the most stable reference gene (*ef1*α) (Supplemental Figure 1), and calibrated with the mean of delta Ct (dCt) of all samples according to the $\Delta\Delta$Ct value method.

2.4. Antibodies

The immunopurified anti-Cyp19a1a antibody was purchased from AnaSpec (Fremont, CA, USA distributed in France by Tebu-bio; reference number: 55474s). According to the manufacturer, this antibody was raised in rabbit against a synthetic peptide derived from the C-terminal region of the zebrafish Cyp19a1a protein (GenBank accession # NP_571229). The manufacturer provided specificity data in zebrafish by Western-blots: only one band was detected and this band disappeared when blocked by the peptide used for immunization. The immunopurified anti-Cyp19a1b antibody used in this study was raised in rabbit against the peptide CNSNGETADNRTSKE, corresponding to the last 15 residues (amino acids 497-511) of the zebrafish Cyp19a1b sequence (AF183908). The immunopurified anti-Cyp17a1 antibody was also raised in rabbit against two synthetic peptides (AFADYSSTWKFHRK and KVRADWEKSPLMQHC) corresponding to the amino acids 126-139 and 505-519 respectively, of the zebrafish Cyp17a1 sequence (AAP41821). These two antibodies have been shown to be specific for the Cyp19a1b protein in zebrafish brain (Menuet *et al.*, 2005; Vosges *et al.*, 2010), and for the Cyp17a1 protein in zebrafish testis (De Waal *et al.*, 2009; Hinfray *et al.*, 2011). In addition, the specificity of the Cyp19a1b antibody in zebrafish testis, was assessed by Western blotting as previously described for Cyp17a1 (De Waal *et al.*, 2009). The Cyp19a1b antibody specifically recognized a single band of about 50 kDa. The observed size of protein is in agreement with those reported in the literature for zebrafish

(Menuet *et al.*, 2005) and with the deduced molecular weight based on the amino-acid sequence (Supplemental Figure 2).

2.5. Histology

Testes were fixed in Bouin's fluid for 48 hours. After fixation, samples were dehydrated in ethanol and embedded in paraffin, according to conventional procedures. Testis were longitudinally sectioned at 5 µm and stained with haematoxylin/eosin or processed in immunohistochemistry as described below.

Morphometric analysis of testis of control and E_2-exposed fish was conducted according to Feitsma *et al.* (2007) with some modifications. Volume fractions of various testicular tissue components were determined as the area of cysts containing cells normalized by the total area of testis analyzed to obtain a percentage of total tissue for each component. These volume fractions were determined for each of the following testicular components: type A spermatogonia, type B spermatogonia, spermatocytes, spermatids, spermatozoa and others (somatic cells, blood and lymphatic vessels, connective tissue and empty spaces). Four testicular sections (~ 80 µm apart from each other) were analyzed for each fish (N = 6 per condition).The weight (mg) of each testis component was determined as the product of the volume fraction (%) and the testis weight. These quantifications were blindly performed without *a priori* knowledge of the specimen identity.

2.6. Immunohistochemistry

Sections were dewaxed and rehydrated, and antigens were unmasked for 3 hours at 80°C in EDTA buffer (1mM, pH 8.5). Tissue sections were then incubated for 1 hour in a blocking solution (PBS containing 0.2% Triton X-100 and 1% milk powder). Incubation with primary antibodies (anti-Cyp19a1a, Cyp19a1b or anti-Cyp17a1) was performed overnight (1:300 (anti-Cyp19a1b or anti-Cyp17a1) or 1:600 (anti-Cyp19a1a) with 0.5% milk powder in PBS) at ambient temperature. After rinsing, sections were incubated for 1h30 with a goat anti-rabbit

antibody coupled to AlexaFluor® 594 (Invitrogen, Praisley, UK) 1:200 with 0.5% milk powder in PBS. The specificity of the staining was controlled by processing adjacent sections without primary antibody or with primary antibody pre-adsorbed with the peptides (100 µg/ml) for Cyp19a1b and Cyp17a1. In these negative controls no cross-reactivity was seen for any of the three antibodies (data not shown). For immunolocalization experiments in non-exposed fish from our breeding unit, and of DMSO- and E2-exposed fish, 6 adult male zebrafish were used.

2.7. Fluorescence analysis

Immunofluorescence was observed with a Zeiss AxioImager.Z1 fluorescence microscope equipped with an AxioCam Mrm camera and combined with an ApoTome (Zeiss GmbH, Göttingen, Germany). Micrographs were taken using the Axiovision Imaging software. All micrograph presented were series of optical sections along the z-axis that were acquired at 0.25 to 0.45 µm intervals with the 20x and 40x objectives and projected in a single image (maximum-intensity projection). Fluorescence pictures of testes of DMSO- or E_2-exposed fish were all series of 20 optical sections acquired with the same time of exposure, however, the time of exposure was adjusted for each antibody used.

Quantification of fluorescence of DMSO- or E_2-exposed fish testes was performed on micrographs taken with the same magnification (20x objective) and time of exposure for a given antibody for all individuals. Fluorescence was measured as integrated density (IntDen, ImageJ software), i.e. the sum of the gray-values of all the pixels in the region of interest. For Cyp19a1, the IntDen data were then normalized to the total area of testis tissue on the micrograph. For Cyp17a1, the IntDen data were normalized to the area occupied by interstitial cells (including Leydig cells) or germ cells. All results were expressed as percentage of their respective controls. For each antibody, 4 micrographs per individual and 4 individuals per treatment were analyzed. Since no statistically significant difference of

fluorescence quantification was observed between DMSO-exposed fish at 7 and 14 days for any of the three antibodies, all the DMSO-exposed fish (7 and 14 days) were pooled.

2.8. Statistical analysis

All data presented are mean ± standard deviation (SD; Fig. 6) or standard error of the mean (SEM; Fig. 7-9) as indicated in the legend of each figure. Data of the morphometric analysis of spermatogenesis (Fig. 6) and of the quantification of fluorescence (Fig. 7-9) were statistically analyzed using the Kruskal-Wallis non-parametric test followed by the Mann-Whitney U-test (SPSS software, IBM Incorporation, New York, USA). The qPCR data (Fig. 2) were analysed by one way ANOVA followed by the Dunnett's multiple comparison test, in which the Egfp-positive cells served as reference group, which was compared to either the Egfp-negative cells or the Egfp-positive cells after differential plating. Differences between groups were considered to be significant if $p < 0.05$.

3. Results

3.1. Immunolocalization of Cyp19a1 and Cyp17a1 proteins in mature zebrafish testis

By using our specific anti-Cyp19a1 and anti-Cyp17a1 zebrafish antibodies, we localized for the first time the cellular sites of expression of the Cyp19a1a and Cyp19a1b enzymes in the zebrafish testis and confirmed and detailed the cellular sites of expression of the Cyp17a1 protein previously described (De Waal *et al.*, 2009; Hinfray *et al.*, 2011).

Until now, information on the identity of aromatase producing cells in the fish testis was scarce. In our study, Cyp19a1a protein was detected in the cytoplasm of Leydig cells (Fig 3A, B) and in all types of germ cells of zebrafish testis (Fig 3A, C-F). Cyp19a1b protein was detected in germ cells of zebrafish testis but was absent from interstitial cells (Fig.4). Immunoreactivity was found in the cytoplasm of spermatogonia (Fig.4A, B), spermatocytes (Fig.4A, C) and spermatids (Fig.4A, D) while spermatozoa remained unlabeled (Fig.4A, E).

Androgens also play an important role in spermatogenesis and Cyp17 is a key enzyme for androgen synthesis. Cyp17a1 protein was detected in both germ cells and interstitial cells (Fig.5). Labelling representing Cyp17a1 protein was observed in the cytoplasm of Leydig cells (Fig.5A, B), spermatogonia (Fig.5A, C), spermatocytes (Fig.5A, D), spermatids (Fig.5A, E) and spermatozoa (Fig.5A, F).

3.2. Expression of cyp19a1 and cyp17a1 genes in testicular germ cells enriched fractions

Only germ cells express Egfp in *vasa::egfp* transgenic animals. Still, the Egfp-positive cell fraction isolated by FACS directly after preparing the cell suspension clearly contained Sertoli cells as shown by the high levels of *gsdf* expression (Fig.2D). We hypothesised that this observation reflected a re-association of germ with Sertoli cells after preparing the cell

suspension and until the start of the FACS procedure. We speculated that differential plating, in order to deplete somatic cells from the suspension, may reduce the contamination. Indeed, two days after differential plating, when FACS-harvesting Egfp-positive cells that floated freely or were only loosely attached, Sertoli cell marker gene expression was clearly reduced in the Egfp-positive fraction (Figs.1, 2D). Further gene expression analysis showed that zebrafish *cyp19a1a* expression was equally found in the somatic (Egfp-negative) and germ cell-enriched fractions (Egfp-positive, floating or loosely attached cells sorted after differential plating) (Fig.2D). A slight increase in *cyp19a1a* mRNA levels after reducing Sertoli cell contamination, similar to the change seen as regards *piwil2* mRNA levels, supports the view that *cyp19a1a* is indeed expressed by germ cells also. Moreover, a clear signal for *cyp19a1a* expression in the Egfp-negative fraction (immediately sorted after preparing the cell suspension) and hence a pattern different from the Sertoli cell gene *gsdf* suggests that *cyp19a1a* expression in the germ cell-free fraction is largely attributable to Leydig cells.

cyp17a1 expression was detected in both somatic (Egfp-negative) and germ cell-enriched cell fractions (Egfp-positive sorted after differential plating; Fig.2D). However, somatic *cyp17a1* mRNA seems to dominate quantitatively over germ cell associated expression, and appears associated rather with Leydig than with Sertoli cells, since its expression pattern was similar to Leydig cell genes (*insl3* and *star*) in the Egfp-negative fraction (Fig.2D). Moreover, when reducing Sertoli cell numbers by differential plating, *cyp17a1* mRNA levels increased in the germ cell enriched fraction. The latter observation also shows that *cyp17a1* mRNA is present in germ cells as well.

3.3. Effect of œstradiol exposure on spermatogenesis and Cyp19a1 and Cyp17a1 protein synthesis in mature zebrafish testis

In vivo exposure to 10nM of E_2 either for 7 or 14 days had no effect on the body weight of exposed fish (data not shown), while a decrease in gonad weight and GSI was observed.

The gonad weight significantly decreased from 9.4 ± 2.2 mg to 3.3 ± 0.3 mg after 14 days of E_2 exposure and the GSI was significantly reduced from 1.8 ± 0.3 % to 1.4 ± 0.1 % after 7 days and from 2.2 ± 0.4 % to 1.0 ± 0.0 % after 14 days.

Quantitative morphometric analysis of zebrafish testis tissue samples collected after 7 and 14 days of E_2 exposure revealed clear, statistically significant shifts in the relative proportion of the different germ cell types (Fig.6A, B). After 7 days, a decrease of spermatocytes to 60 % of the control levels was observed (Fig.6A). At 14 days of E_2 exposure, the main changes in the germ cell populations revealed by morphometric analysis were the increase of the mass of type A spermatogonia (~ 175 % of the control level) and the decrease of the mass of type B spermatogonia (~ 32 % of the control level), of spermatocytes (~ 11 % of the control level), of spermatids (~ 25 % of the control level) and of spermatozoa (~ 43 % of the control level) (Fig.6B).

We then examined the effect of E_2 exposure on the testicular synthesis of Cyp19a1a and b and Cyp17a1 proteins. Immunohistochemistry experiments with anti-Cyp19a1 antibodies revealed that in control and E_2-exposed fish testis (7 and 14 days), Cyp19a1a immunoreactivity was observed in Leydig cells and all types of germ cells (Fig.7A), while Cyp19a1b was detected in spermatogonia, spermatocytes and spermatids (Fig.8A). Moreover, evaluation of Cyp19a1a and Cyp19a1b fluorescence showed no statistically significant difference between control and E2-exposed fish whatever the duration of exposure (7 and 14 days) (Fig 7B and 8B).

Contrary to aromatases, E_2 exposure (7 and 14 days) led to a strong inhibition of Cyp17a1 protein immunolabelling in zebrafish testis (Fig.9A, B). Interestingly, our study revealed that this inhibition occurred only in Leydig cells and not in germ cells. Indeed, after 7 and 14 days of exposure, Cyp17a1 fluorescence decreased drastically in interstitial cells including Leydig cells from testis of E_2-exposed fish (Fig.9A, B).

4. Discussion

Localization of Cyp19a1 and Cyp17a1 proteins in mature zebrafish testis

In zebrafish, the two *cyp19a1* genes were known to be expressed in the mature testis (Sawyer *et al.*, 2006; Hinfray *et al.*, 2011). Using immunohistochemistry experiment, we have localized here the cellular sites of expression of Cyp19a1 enzymes in the zebrafish testis. Cyp19a1a is expressed in both, Leydig and germ cells, as shown by two independent techniques, i.e. immunocytochemistry and analysis of gene expression in FACS-sorted testicular cell fractions. Using immunohistochemistry, Cyp19a1b, on the other hand, was detected only in germ cells. In the serially sex changing gobiid fish, *Trimma okinawae*, expression of the *cyp19a1a* and *cyp19a1b* genes was detected in the testis by RT-PCR; *in situ* hybridization detected *cyp19a1a* mRNA in interstitial cells of the testis whereas *cyp19a1b* mRNA was not detected (Kobayashi *et al.*, 2004). In sea bass, RT-PCR experiments revealed the presence of *cyp19a1a* in testicular germ cells (Vinas and Piferrer, 2008). In rainbow trout, while some reported no aromatase immunoreactivity in the testis (Kobayashi *et al.*, 1998), others showed aromatase immunolabeling in germ, Leydig, and Sertoli cells although the use of an antibody against human placental aromatase did not allow differentiating the two aromatase isoforms (Kotula-Balak *et al.*, 2008). In different mammalian species, aromatase protein and enzymatic activity was demonstrated in testicular germ cells (for review see (Carreau, 2001). Despite certain differences between studies, the localization of aromatase in testis together with the known presence of oestrogen receptors in somatic and germ cells of fish testis (Bouma and Nagler, 2001; Wu *et al.*, 2001; Menuet *et al.*, 2002; Vinas and Piferrer, 2008), suggests the involvement of oestrogens in both major testicular functions, steroid production and spermatogenesis. Indeed, in fish, there is evidence to indicate that spermatogonial stem cell renewal is regulated by oestradiol (Miura *et al.*, 1999, 2003).

Like Cyp19a1a, Cyp17a1 protein and gene expression was localized both in Leydig and germ cells of zebrafish testis, with a quantitative prevalence in Leydig cells. Other studies previously reported that Cyp17a1 is expressed in Leydig cells (Kobayashi *et al.*, 1998; Zhou *et al.*, 2007; De Waal *et al.*, 2009; Hinfray *et al.*, 2011). Recent studies also showed that the enzymes required for the production of the 11-oxygenated androgens typically found in fish (Cyp17 and Cyp11b) are present in male germ cells (Vinas and Piferrer, 2008; Sreenivasulu and Senthilkumaran, 2009; Zhang *et al.*, 2010). Androgens are required for spermatogenesis *ex vivo* (Miura *et al.*, 1991) or *in vivo* (De Waal *et al.*, 2009), and recently, a role for the androgens also in milt hydration or in providing the proper environment for sperm maturation/conservation has been hypothesized in fish (Rolland *et al.*, 2009).

Effect of oestradiol exposure on spermatogenesis and Cyp19a1 and Cyp17a1 protein synthesis in zebrafish testis

In the present study, exposure of adult male zebrafish for 7 and 14 days to 10 nM of E_2 led to alterations in spermatogenesis. Indeed, fish exposed to E_2 for 14 days presented testis with more type A spermatogonia and less type B spermatogonia, spermatocytes, spermatids and spermatozoa. The effects on the accumulation of spermatogonia type A and the reduced mass of spermatogonia type B, spermatocytes and spermatids were similar to findings in a previous study where zebrafish were exposed for 21 days to the same concentration of E_2 (De Waal *et al.*, 2009). However, in the latter study, we did not observed a decrease in spermatozoa or others (i.e. somatic cells, blood vessels, connective tissue, empty space). At present, we do not have an explanation for these differences but it cannot be excluded that certain differences in the histological analysis techniques (e.g. glutaraldehyde *versus* Bouin's fixative; plastic *versus* paraffin embedding, or area of cysts *versus* intersection grid for the calculation of volume fractions) may have contributed to the slight differences.

Immunohistochemistry experiments with anti-Cyp19a1 antibodies showed no evidence for a difference between control and E_2-exposed fish whatever the duration of exposure (7 and 14 days). Short term exposures of fathead minnows and medaka to oestrogens from 5 to 500 ng/L during either 7 or 14 days had no effect on *cyp19a1a* gene expression in the testis (Filby *et al.*, 2006; Zhang *et al.*, 2008). In zebrafish, an exposure for 7 days to 10nM E_2 had no effect on testicular *cyp19a1a* gene expression (Hinfray N & Brion F 2011, unpublished observations) whereas it strikingly inhibited ovarian *cyp19a1a* gene expression (Hinfray *et al.*, 2006). For aromatase B, some studies showed that testicular *cyp19a1b* gene expression in fathead minnow increased following a 14 day long exposure to oestrogens (Halm *et al.*, 2002; Filby *et al.*, 2006). In zebrafish, no effect was observed on testicular *cyp19a1b* gene expression after 5 days of exposure to 1nM E_2 (Sawyer *et al.*, 2006), or after 7 days of exposure to 10nM E_2 (Hinfray N & Brion F 2011, unpublished observations). Neural *cyp19a1b* gene expression, on the other hand, is oestrogen-sensitive in zebrafish, and it was only in glial or neuro-glial cells that stimulation of the zebrafish *cyp19a1b* gene promoter by oestrogens could be demonstrated (Diotel *et al.*, 2010). Altogether, these reports are in agreement with the absence of effect of E_2 on testicular aromatases in our study.

Contrary to aromatases, E_2 exposure (7 and 14 days) led to a strong inhibition of Cyp17a1 protein immunolabelling in zebrafish testis. While this observation is consistent with the oestrogen-induced decrease in *cyp17a1* mRNA levels reported in fish testis (Govoroun *et al.*, 2001; Baron *et al.*, 2005; Brion *et al.*, 2008; Zhang *et al.*, 2008; De Waal *et al.*, 2009), our study moreover revealed that this inhibition occurred only in Leydig cells but not in germ cells. Indeed, after 7 and 14 days of exposure, a drastic inhibition of Cyp17a1 fluorescence was observed in Leydig cells from testis of E_2-exposed fish. In the present study, perturbations of spermatogenesis started to become evident after 7 days and were very clear after 14 days of E_2-exposure. It was stated previously that oestrogen treatment induced an androgen insufficiency in zebrafish testis resulting in an interruption of spermatogenesis (De Waal *et al.*, 2009). Indeed, in zebrafish, exposures to oestrogens for 7 days led to inhibition of *cyp17a1*

expression (Brion *et al.*, 2008; De Waal *et al.*, 2009) and a decrease of testicular 11-ketotestosterone production (De Waal *et al.*, 2009) probably related to the disrupted spermatogenesis observed after 14 days of E_2-exposure. The present results demonstrate that down-regulation of Cyp17a1 and hence of androgen synthesis occurs specifically in Leydig cells, thereby becoming a major molecular and cellular event in the oestrogen-induced perturbation of zebrafish spermatogenesis. Our findings also raise questions as regards the regulation of expression of steroidogenic enzymes in germ cells. It is unclear at present why Cyp17a1 synthesis was not down-regulated by E_2 in germ cells, in contrast to Leydig cells. It can be hypothesized that only in Leydig cells there is an appropriate cellular context for transcriptional inhibition by E_2. Another hypothesis is that E2 exerts a negative feedback on the brain/pituitary system, resulting in a reduced gonadotropin release, and consequently a fading stimulation of *cyp17a1* gene expression and Cyp17a1 synthesis in Leydig cells which, in contrast to germ cells, express receptors for both Fsh and Lh in fish (Garcia-Lopez *et al.*, 2010).

Our study also raises questions as regards the functionality of the steroidogenic enzymes in germ cells. Steroid metabolising enzyme activity in spermatozoa has been demonstrated in different vertebrates, ranging from mammals (for review see (Carreau, 2001; Carreau *et al.*, 2006) to fish (Sakai *et al.*, 1989; Asahina *et al.*, 1990; Asahina *et al.*, 1994). In zebrafish, the expression of both aromatases and Cyp17a1 in germ cells suggests that these cells can produce locally androgens and oestrogens *de novo*. However, we do not know if germ cells possess all the steroidogenic enzymes allowing the entire steroidogenic process to occur locally or if germ cell steroid production depends on the supply of steroidal substrates from other (probably Leydig) cells for androgen, and possibly also for the subsequent oestrogen production.

In summary, our study provides new data on the cellular localization and expression of Cyp19a1 and Cyp17a1 in the zebrafish testis. Cyp19a1b is synthesized only by germ cells

(spermatogonia, spermatocytes and spermatids) while Cyp19a1a and Cyp17a1 are synthesized by Leydig cells and all types of germ cells. As regards Cyp17a1, both protein and mRNA seem to be quantitatively dominating in Leydig cells. Further, we demonstrate that E_2 exposure had no effect on the synthesis of these proteins in germ cells but suppressed Cyp17a1 synthesis in Leydig cells after 7 and 14 days of exposure. Since oestrogen-induced androgen insufficiency is associated with a Leydig cell-specific down-regulation of Cyp17a1, the latter can be identified as a major event in altering testicular steroidogenesis and consequently spermatogenesis. The physiological predominance of Leydig cell expression of steroidogenic enzymes moreover suggests that germ cell steroid production may have to be fuelled by precursors from Leydig cells. Further studies are needed to elucidate the functionality of steroidogenic enzymes in germ cells and their potential role in the testicular physiology.

5. Declaration of interests

The authors declare there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Figure caption

Figure 1: Differential Plating Method. **A:** Scheme showing the differential plating method adapted for zebrafish testicular cell suspension from Luo *et al*. (2006). A total cell suspension (fraction 1) was obtained from zebrafish testes, and harvested in cell culture medium (L-15). After 2 days of culture, only somatic cells with adhesive properties (Sertoli cells, blue triangular symbols; Leydig cells, yellow oval symbols) adhere to the bottom of the plate (Fraction 2), while germ cells (non-adherent cells; green symbols reflecting their transgenic *vasa::egfp* state) remain floating, or attach loosely to the firmly attached Sertoli cells. After an extensive washing step, it is possible to remove floating germ cells and also those germ cells weakly attached to the somatic cells, leaving the adherent somatic cells (Sertoli cells, Leydig cells, fibroblast and others – **Fraction 3**) at the bottom of the plate. This method is called differential plating due to the different adhesive properties of the different testicular cell types. **B,C. Fraction 1**. Floating cells: adherent and non-adherent cells which can be isolated or in groups. GC (germ cell). **D,E. Fraction 2**. Somatic cells (SC) with adhesive properties at the bottom of the plate. Arrow shows interaction between somatic cells and germ cells (GC). **F,G. Fraction 3**. After extensive washing, somatic adherent cells (SC) remain attached to the bottom of the plate, while the floating and the weakly attached germ cells were removed.

Figure 2: A: FACS dot plot profile obtained from wild-type (left) and *vasa::egfp* transgenic zebrafish (right) testicular cell suspension. Each dot represents one single event (one cell), x axis represents the intensity of Egfp (more to the right – more Egfp, while more to the left less or no Egfp), y axis represents another filter which was used to evaluate autofluorescence. The percentage of Egfp positive events in the total testicular cell suspension for wild-type (left; 0.07%) and *vasa::egfp* transgenic (right; 71.53%) zebrafish is indicated in the selected area. The selected area in the right graph shows the Egfp positive population which was harvested by FACS; the rest consists the Egfp negative fraction. **B,C.**

Testicular cell suspension from *vasa::egfp* transgenic animals before FACS analysis, showing Egfp positive and negative cells. **B:** DIC microscopy. **C:** Fluorescence microscopy. **D:** Relative mRNA expression of several testicular genes (*gsdf*, *insl3*, *cyp17a1*, *cyp19a1a*, *star*, and *piwil2*) in the different cell fractions: Egfp + (dark green; immediately sorted after cell suspension; n=4), Egfp – (gray; immediately sorted after cell suspension; n=2), and Egfp + sorted after differential plating (after 2 days of culture; light green; n=2) to reduce somatic cell numbers and to enrich germ cells. Data are expressed as mean ± standard error of the mean (SEM). The level of statistical significance between the reference group (Egfp +) and the other groups (Egfp – and EGFP + sorted after differential plating) are indicated by * ($p<0.05$), ** ($p<0.01$), *** ($p<0.005$), or ns (not significant).

Figure 3: Immunolocalization of Cyp19a1a protein in testis of adult zebrafish (*Danio rerio*). (A) Representative micrograph (from n = 6 fish) of Cyp19a1a immunoreactive cells (red). Arrows indicate Leydig cells. Higher magnifications show that immunoreactivity was localized in the cytoplasm of Leydig cells (B), spermatogonia (C), spermatocytes (D), spermatids (E) and spermatozoa (F). sc: spermatocytes, sg: spermatogonia, st: spermatids, sz: spermatozoa. White: Hoechst staining. Scale Bars = 50 µm (A) and 10 µm (B-F).

Figure 4: Immunolocalization of Cyp19a1b protein in testis of adult zebrafish (*Danio rerio*). (A) Representative micrograph (from n = 6 fish) of Cyp19a1b immunoreactive cells (red). Higher magnifications show that immunoreactivity was localized in the cytoplasm of spermatogonia (B), spermatocytes (C), spermatids (D) but not spermatozoa (E). White arrows show the localization of spermatogonia nucleus. *: sectioning artifacts filled with air bubbles. sc: spermatocytes, sg: spermatogonia, st: spermatids, sz: spermatozoa. White: Hoechst staining. Scale Bars = 50 µm (A) and 10 µm (B-E).

Figure 5: Immunolocalization of Cyp17a1 protein in testis of adult zebrafish (*Danio rerio*). (A) Representative micrograph (from n = 6 fish) of Cyp17a1 immunoreactive cells (red). Arrows indicate Leydig cells. Higher magnifications show that immunoreactivity was localized in the cytoplasm of Leydig cells (B), spermatogonia (C), spermatocytes (D), spermatids (E) and spermatozoa (F). sc: spermatocytes, sg: spermatogonia, st: spermatids, sz: spermatozoa. White: Hoechst staining. Scale Bars = 50 µm (A) and 10 µm (B-F).

Figure 6: Zebrafish spermatogenesis after exposure to 10 nM E_2 *in vivo*. Morphometric analysis (n = 6 fish per condition) of zebrafish testis sections (n = 4 per fish) after 7 days (A) and 14 days (B) of E2 exposure, presenting data as mass (mg) of testicular cell types. Results are expressed as mean ± standard deviation (SD). SgA: type A spermatogonia, sgB: type B spermatogonia, sc: spermatocytes, st: spermatids, sz: spermatozoa. Bars marked with ***** are significantly different from their respective control (Kruskal-Wallis non-parametric test followed by the Mann-Whitney U-test, $p < 0.05$).

Figure 7: Expression of Cyp19a1a protein in testis of adult zebrafish (*Danio rerio*) after exposure to 10 nM of estradiol (E2). A: Immunolocalization of Cyp19a1a protein**.** Representative micrograph (from n = 6 fish) of Cyp19a1a immunoreactive cells (red) in control fish (DMSO) and E_2-exposed fish after 7 days (E2 7d) and 14 days (E2 14d). Immunoreactivity was localized in the cytoplasm of Leydig cells (arrows), spermatogonia, spermatocytes, spermatids and spermatozoa in all fish. Insets show immunoreactive Leydig cells at higher magnification. White: Hoechst staining. Scale Bars = 50 µm. Scale bars in insets = 10 µm. **B**: Quantification of Cyp19a1a fluorescence. Results are expressed in percentage of the DMSO-exposed fish fluorescence and as mean ± standard error of the mean (SEM). Number of fish analyzed: n = 8 for control fish and n = 4 for E2-exposed fish.

No significant difference was observed whatever the condition (Kruskal-Wallis non-parametric test followed by the Mann-Whitney U-test, $p < 0.05$).

Figure 8: Expression of Cyp19a1b protein in testis of adult zebrafish (*Danio rerio*) after exposure to 10 nM of estradiol (E2). A: Immunolocalization of Cyp19a1b protein**.** Representative micrograph (from n = 6 fish) of Cyp19a1b immunoreactive cells (red) in control fish (DMSO) and E_2-exposed fish after 7 days (E2 7d) and 14 days (E2 14d). Immunoreactivity was localized in the cytoplasm of spermatogonia, spermatocytes and spermatids in all fish. White: Hoechst staining. Scale Bars = 50 µm. **B**: Quantification of Cyp19a1b fluorescence. Results are expressed in percentage of the DMSO-exposed fish fluorescence and as mean ± standard error of the mean (SEM). Number of fish analyzed: n = 8 for control fish and n = 4 for E2-exposed fish. No significant difference was observed whatever the condition (Kruskal-Wallis non-parametric test followed by the Mann-Whitney U-test, $p < 0.05$).

Figure 9: Expression of Cyp17a1 protein in testis of adult zebrafish (*Danio rerio*) after exposure to 10 nM of estradiol (E2). A: Immunolocalization of Cyp17a1 protein**.** Representative micrograph (from n = 6 fish) of Cyp17a1 immunoreactive cells (red) in control fish (DMSO) and E_2-exposed fish after 7 days (E2 7d) and 14 days (E2 14d). Immunoreactivity was localized in the cytoplasm of Leydig cells (arrows) and germ cells in control fish. After both 7 and 14 days of E2-exposure, Cyp17a1 protein drastically decreased in Leydig cells. Inset in (E2 14d) shows Leydig cells at higher magnification. White: Hoechst staining. Scale Bars = 50 µm. Scale bar in inset = 5 µm. **B**: Quantification of Cyp17a1 fluorescence in interstitial cells and germ cells. Results are expressed in percentage of the DMSO-exposed fish fluorescence for each type of cells (interstitial or germ cells) and as mean ± standard error of the mean (SEM). Number of fish analyzed: n = 8 for control fish and

n = 4 for E2-exposed fish. Fluorescence decreased in interstitial cells (including Leydig cells) after 7 and 14 days of E2 exposure while no effect was measured in germ cells (Kruskal-Wallis non-parametric test followed by the Mann-Whitney U-test, ***: $p < 0.005$).

Supplemental Figure 1: Scatter plot to check the stability of *18S* rRNA and *ef1α* mRNAs as a housekeeping gene in the different cell fractions. Each dot in the scatter plot represents the average Ct-value of duplicate measurements for each cell fraction. Stability was best for *ef1α*.

Supplemental Figure 2: Immunoblot on zebrafish (*Danio rerio*) mature testis Cyp19a1b protein. The anti-Cyp19a1b antibody detected one band of about 50 kDa.

Fig. 1

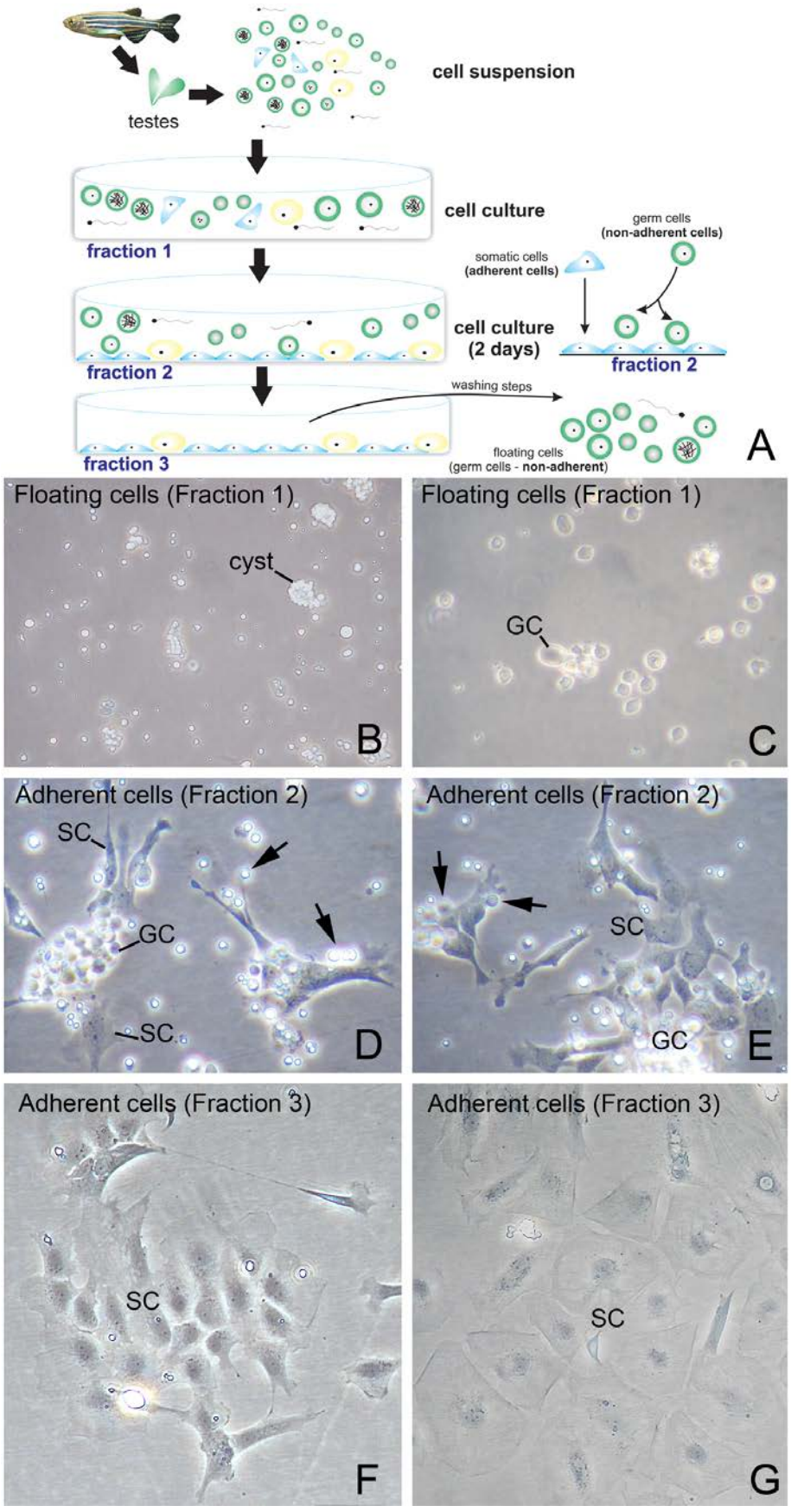

testes
cell suspension
cell culture
fraction 1
germ cells
(non-adherent cells)
somatic cells
(adherent cells)
cell culture
(2 days)
fraction 2
fraction 2
washing steps
fraction 3
floating cells
(germ cells - non-adherent)
A
Floating cells (Fraction 1)
cyst
B
Floating cells (Fraction 1)
GC
C
Adherent cells (Fraction 2)
SC
GC
SC
D
Adherent cells (Fraction 2)
SC
GC
E
Adherent cells (Fraction 3)
SC
F
Adherent cells (Fraction 3)
SC
G

Fig. 2

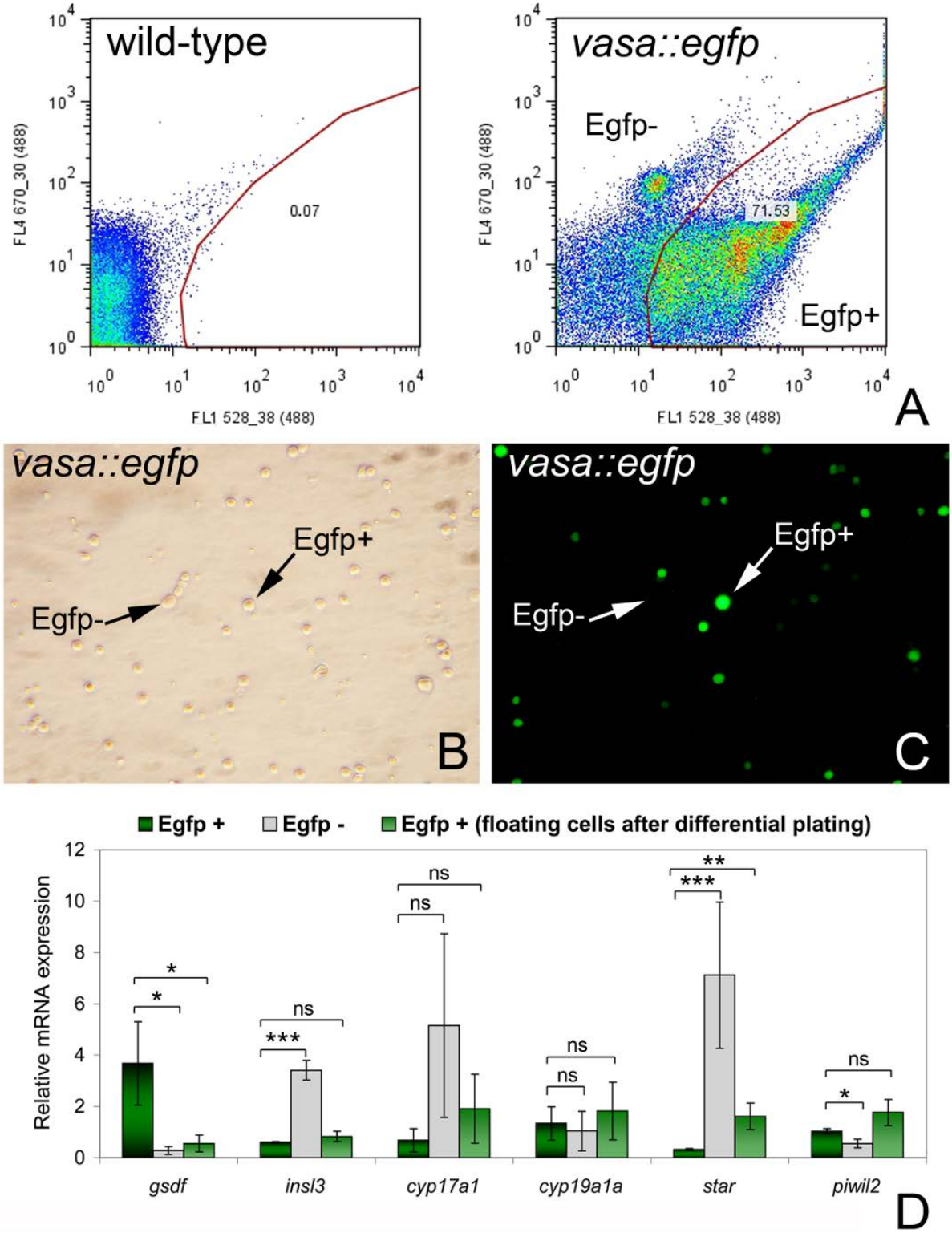

wild-type
0.07
FL4 670_30 (488)
FL1 528_38 (488)
vasa::egfp
Egfp-
71.53
Egfp+
A
vasa::egfp
Egfp+
Egfp-
B
vasa::egfp
Egfp+
Egfp-
C
Egfp +
Egfp -
Egfp + (floating cells after differential plating)
Relative mRNA expression
gsdf
insl3
cyp17a1
cyp19a1a
star
piwil2
D

Fig. 3

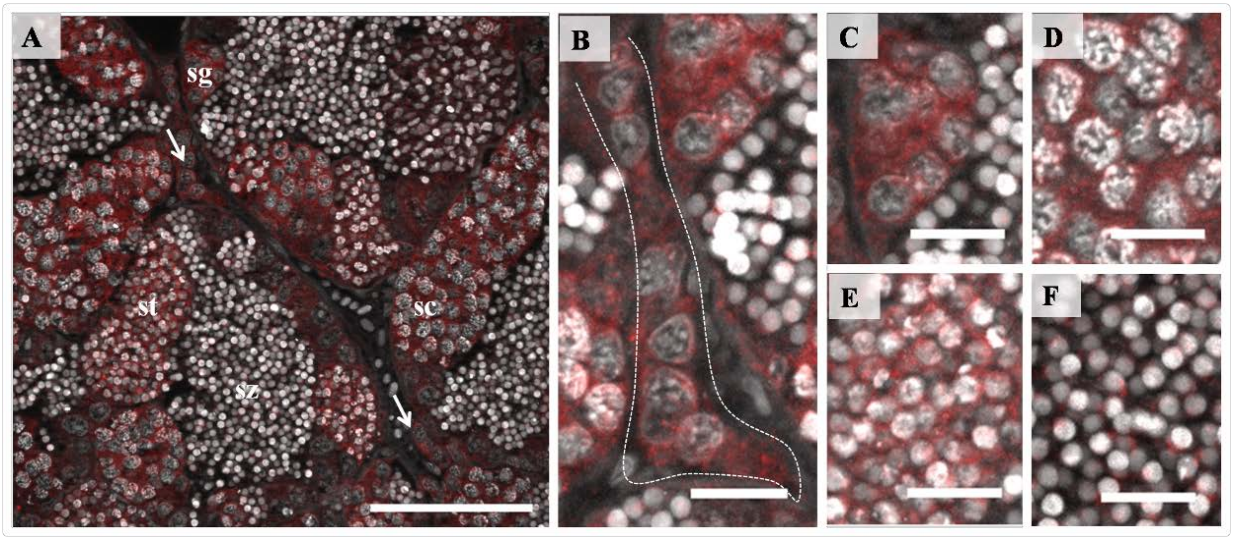


Fig. 4

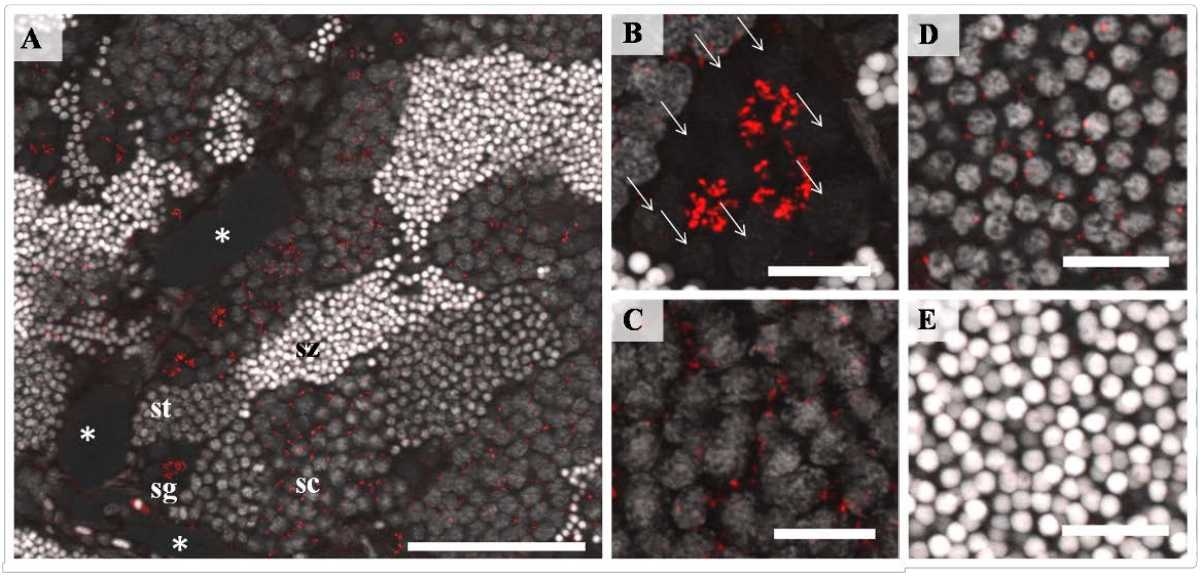


Fig. 5

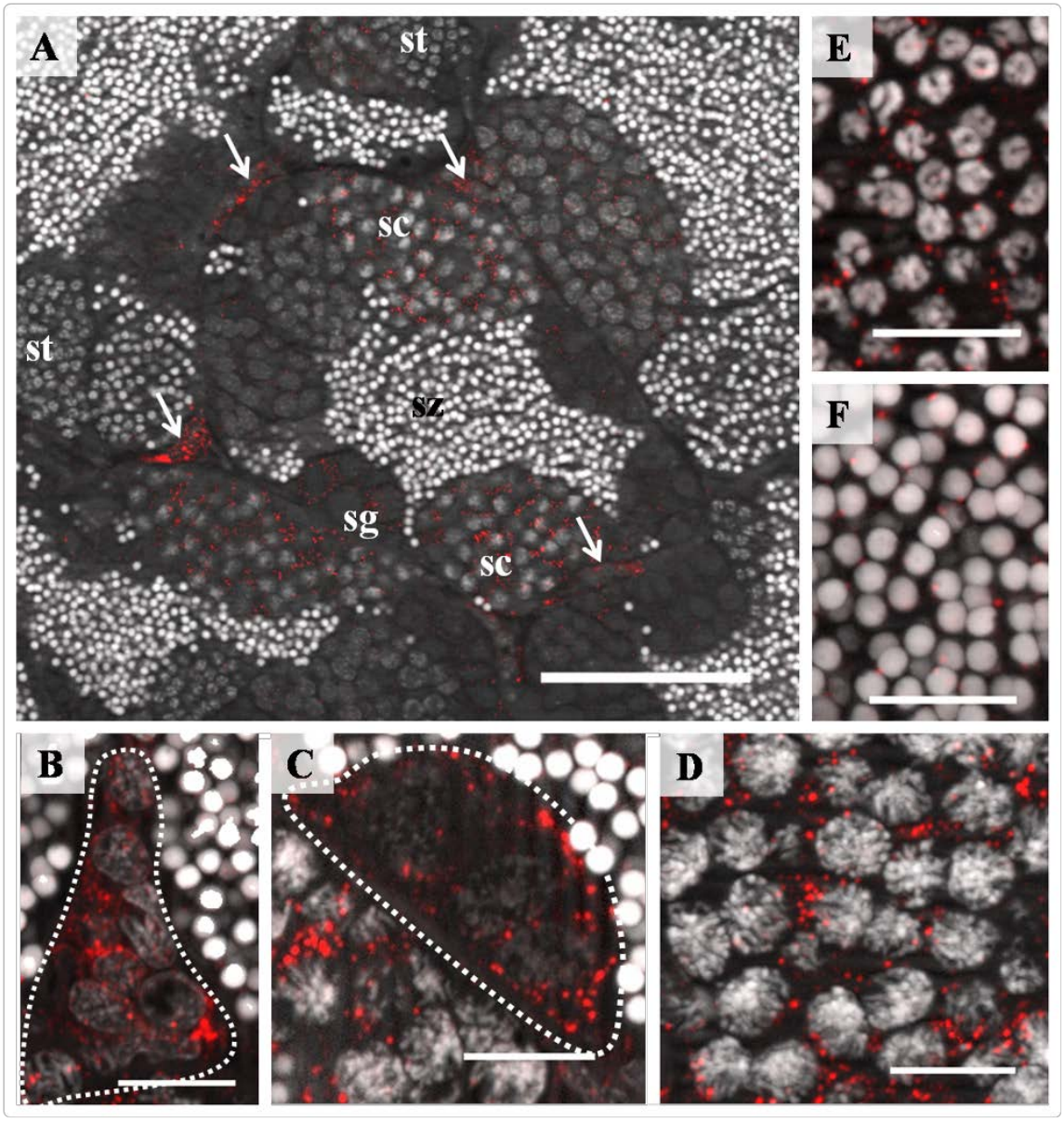


Fig. 6

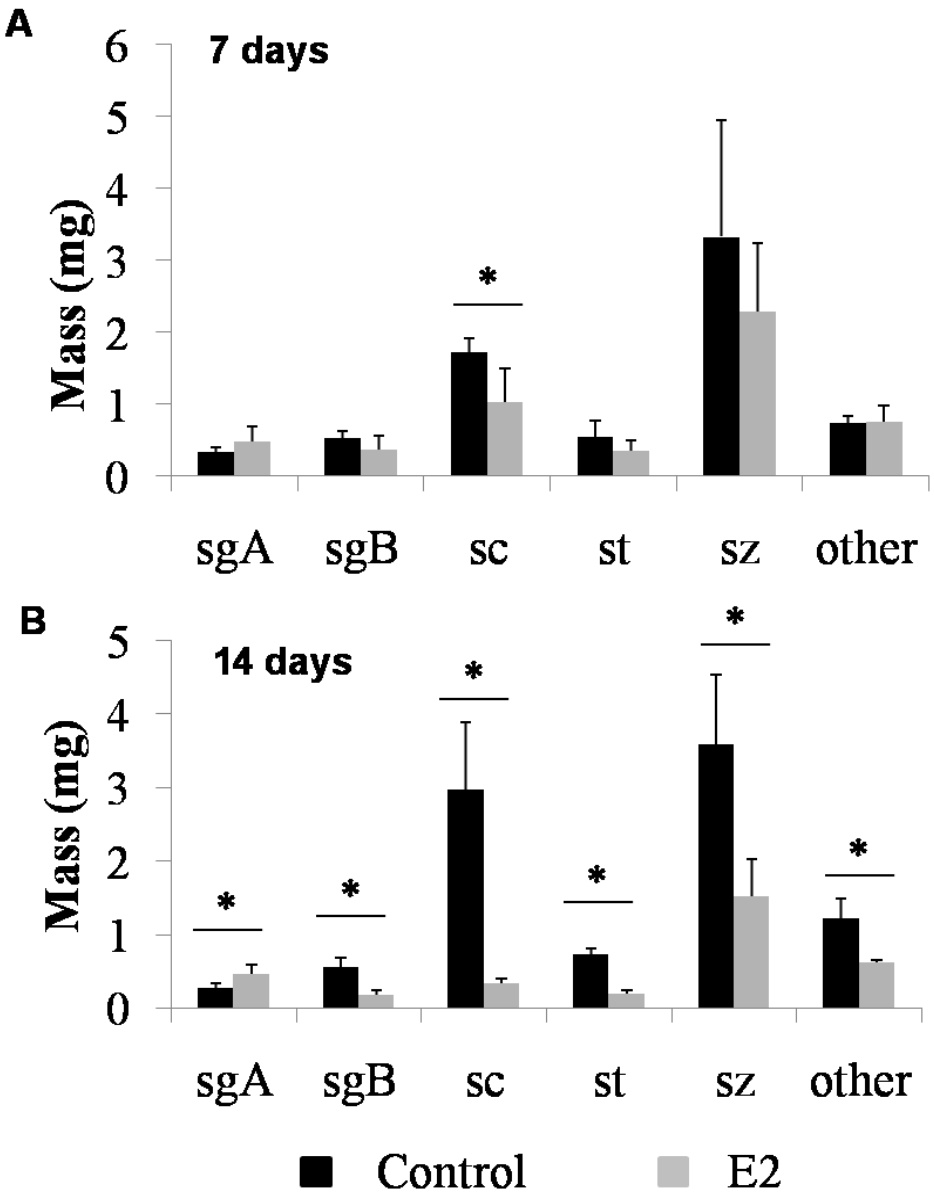

A
7 days
Mass (mg)
0
1
2
3
4
5
6
*
sgA
sgB
sc
st
sz
other
B
14 days
Mass (mg)
0
1
2
3
4
5
*
*
*
*
*
*
sgA
sgB
sc
st
sz
other
Control
E2

Fig. 7

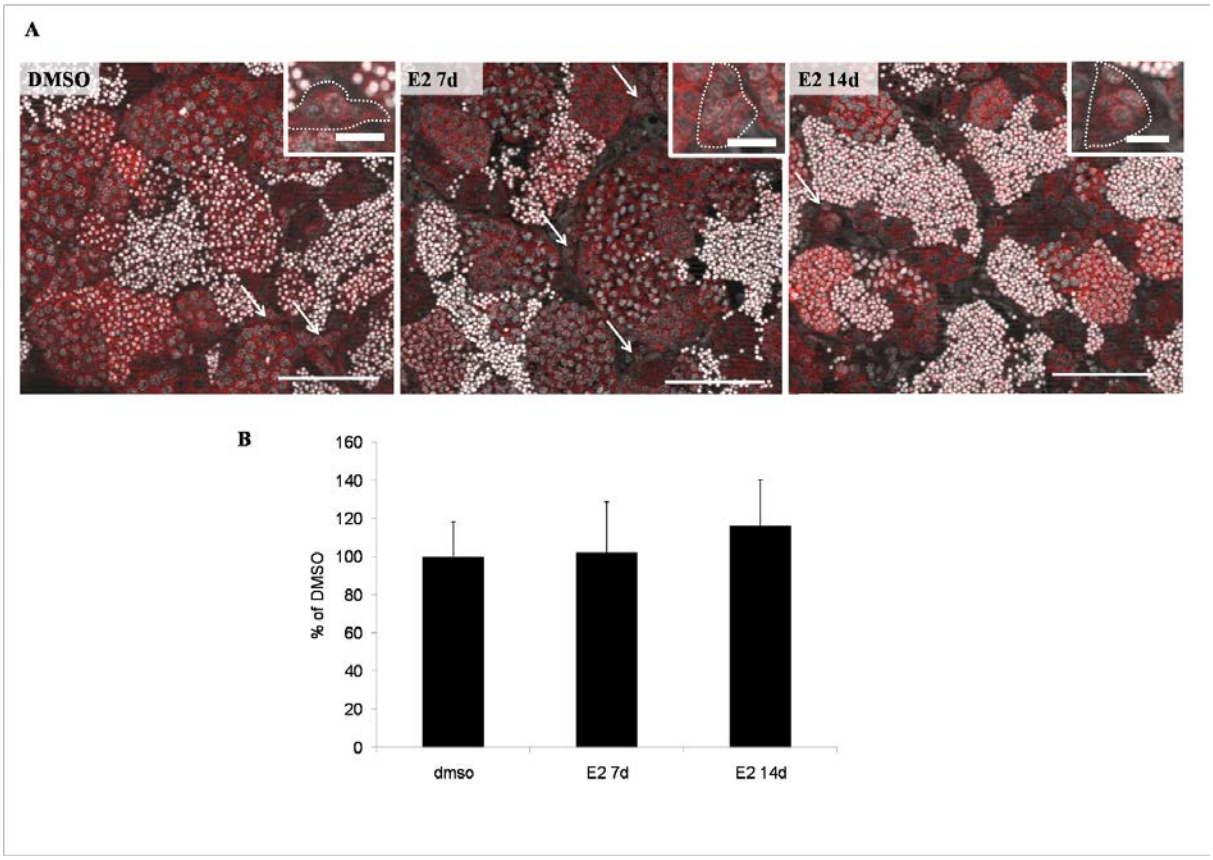

A
DMSO
E2 7d
E2 14d
B
% of DMSO
160
140
120
100
80
60
40
20
0
dmso
E2 7d
E2 14d

Fig. 8

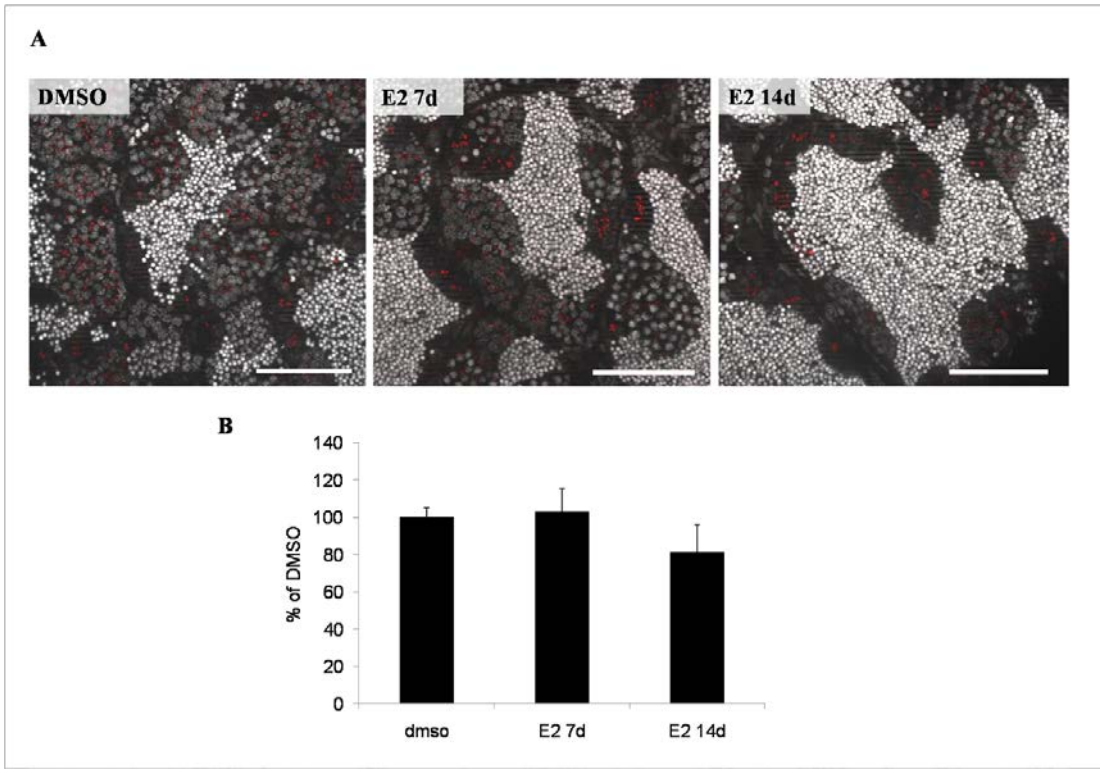
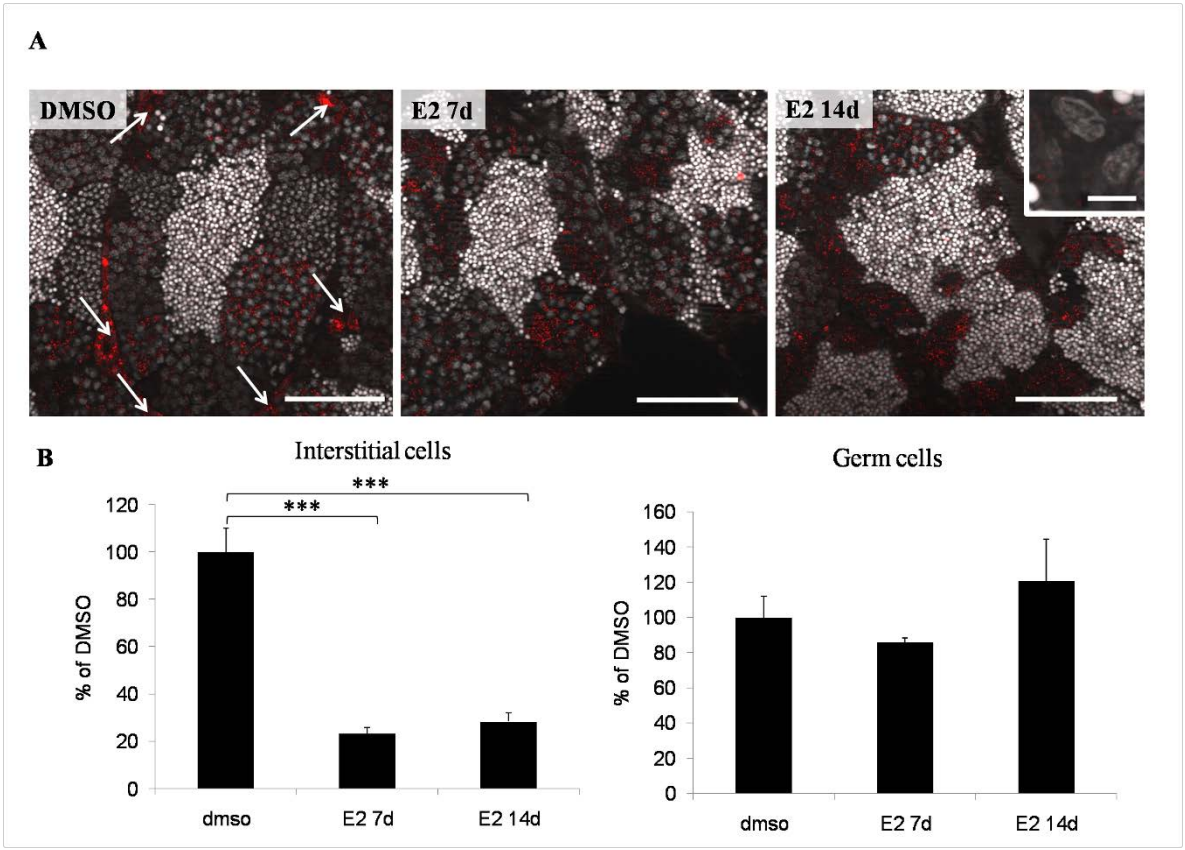

A
DMSO
E2 7d
E2 14d
B
% of DMSO
140
120
100
80
60
40
20
0
dmso
E2 7d
E2 14d

Fig. 9


A
DMSO
E2 7d
E2 14d
B
Interstitial cells
***
***
% of DMSO
120
100
80
60
40
20
0
dmso
E2 7d
E2 14d
Germ cells
% of DMSO
160
140
120
100
80
60
40
20
0
dmso
E2 7d
E2 14d

Supplemental Table 1. Nucleotide sequences for the forward (Fw) and reverse (Rv) primers used for the qPCR reactions.

Target	***Gene Bank Acession no.***	***Primer***	**Nucleotide sequence (5'⟶ 3')**
gsdf	EU378916	Fw	CATCTGCGGGAGTCATTGAAA
		Rv	AGCTTGCCGGAGGACTCTG
insl3	EF685704	Fw	TCGCATCGTGTGGGAGTTT
		Rv	TGCACAACGAGGTCTCTATCCA
cyp17a1	NM_212806	Fw	GGGAGGCCACGGACTGTTA
		Rv	CCATGTGGAACTGTAGTCAGCAA
cyp19a1	NM_131154.2	Fw	GACACCTGGCAGACTGTATTAATCAA
		Rv	CTGTGATGGCATCCTGCAACT
star	AF220435	Fw	CCTGGAATGCCTGAGCAGAA
		Rv	ATCTGCACTTGGTCGCATGAC
piwil2	NM_001080199.3	Fw	TGATACCAGCAAGAAGAGCAGATCT
		Rv	ATTTGGAAGGTCACCCTGGAGTA
ef1α	NM_131263.1	Fw	GCCGTCCCACCGACAAG
		Rv	CCACACGACCCACAGGTACAG
		Probe	FAMCTCCAATTTTGTACACATCCTGAAGTGGCATAMRA

Fig. S1

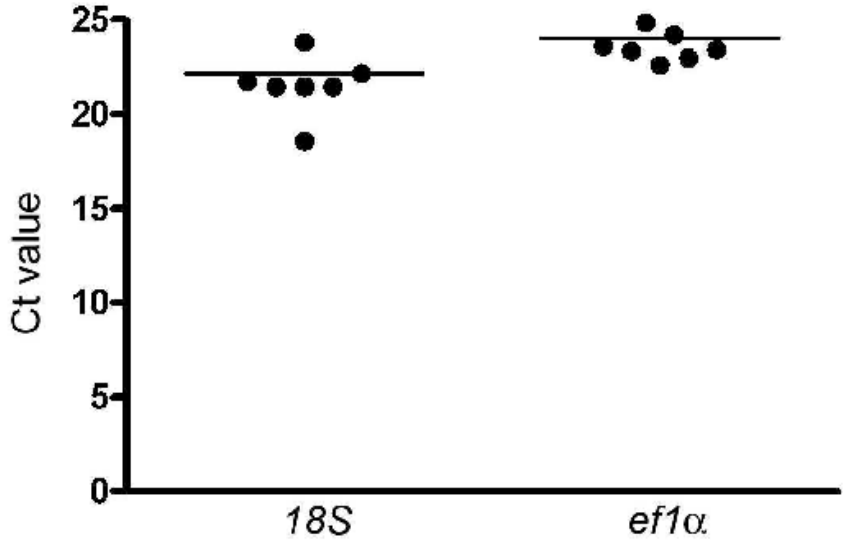


Fig. S2